Separation and characterization of a novel isoenzyme of cyclic nucleotide phosphodiesterase from rat cerebrum

Jun Mukai, Tomoko Asai, Michiko Naka & ¹Toshio Tanaka

Department of Molecular and Cellular Pharmacology, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514, Japan

Anion-exchange chromatography on a Mono-Q column of the supernatant fraction, after ultracentrifugation, from a homogenate of rat cerebrum, prepared under isotonic conditions in the presence of protease inhibitors, yielded a novel isoenzyme of cyclic nucleotide phosphodiesterase (PDE) with properties unlike those of known PDEs. The isoenzyme was insensitive to stimulation by Ca^{2+} /calmodulin and cyclic GMP, and it hydrolyzed both cyclic AMP and cyclic GMP with K_M values of $0.109 \pm 0.008 \mu\text{M}$ and $1.78 \pm 0.04 \mu\text{M}$, respectively. The ratio of V_{max} of hydrolysis of cyclic GMP to that of cyclic AMP was 1.90 ± 0.07 . Nicardipine (PDE I inhibitor), SK&F 94120 (PDE III inhibitor), rolipram (PDE IV inhibitor) and zaprinast (PDE V inhibitor) had very weak inhibitory effects on the PDE activity of the isoenzyme. These results suggest that the isoenzyme is a novel and previously unreported species of PDE, which we tentatively designate PDE VIII.

Keywords: Cyclic AMP phosphodiesterase; phosphodiesterase inhibitors; rat cerebrum; PDE VIII

Introduction Recent information obtained from cloning, primary-sequence, and drug-selectivity studies suggests that there exists a large superfamily of phosphodiesterases (PDEs), which consists of seven distinct families of PDE isoenzymes (Beavo *et al.*, 1990; Michaeli *et al.*, 1993). Many studies have illustrated clear differences among tissues in terms of the distribution of PDE isoenzymes. In a previous paper, the rat cerebrum was shown to be the source of three activities, designated PDE I, PDE II and PDE IV (Nicholson *et al.*, 1989). However, the low resolution of the ion-exchange chromatographic techniques employed in the earlier study failed adequately to fractionate the various isoforms in this tissue. In the present study we separated the PDE isoenzymes in the rat cerebrum by anion-exchange chromatography on a Mono-Q column, and characterized the various PDE activities by both pharmacological and biochemical techniques.

Methods *Isolation of PDE isoenzymes from the rat cerebrum* The study was performed on tissue obtained from adult male rats that had been killed by cervical dislocation. All preparative procedures were performed at 4°C.

Five grams of cerebral cortex were excised from rats and rinsed several times in ice-cold homogenization buffer [Buffer A: Tris-HCl 50 mM (pH 7.5), sucrose 0.25 M, EGTA 0.1 mM, dithiothreitol 0.1 mM, leupeptin 20 μM , soybean trypsin inhibitor 10 $\mu\text{g ml}^{-1}$, pepstatin 10 $\mu\text{g ml}^{-1}$, chymostatin 10 $\mu\text{g ml}^{-1}$ and (*p*-amidinophenyl)methanesulphonyl fluoride hydrochloride 1 μM]. The tissue was gently homogenized, with a Potter S homogenizer, in 8–10 volumes of Buffer A. The homogenate was then centrifuged at 100,000 *g* for 60 min. The resultant supernatant, containing cytosolic PDEs, was filtered through glass wool and then through a cellulose acetate 0.20 μm filter unit (ADVANTEC Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtered supernatant was applied to a Mono-Q HR 5/5 (Pharmacia LKB Biotechnology, Uppsala, Sweden) anion-exchange column (0.5 $\times$ 5 cm) at a flow rate of 0.5 ml min⁻¹, after the column had been equilibrated with Buffer B [Tris-HCl 50 mM (pH 7.5), EGTA 0.1 mM, dithiothreitol 0.1 mM and (*p*-amidinophenyl)methanesulphonyl fluoride hydrochloride 1 μM]. The loaded column was washed with 30 ml of Buffer B. The cytosolic PDE isoenzymes were then eluted with a 100-ml linear

gradient of NaCl (0–0.12 M) in Buffer B, at a flow rate of 0.5 ml min⁻¹. Fractions of 1 ml each were collected.

Analytical procedures PDE activity was measured by a two-step assay (Masuoka *et al.*, 1990). The molecular mass of the novel isoenzyme was determined by gel filtration on Superose 12 HR 10/30 (Pharmacia LKB Biotechnology).

Materials Papaverine hydrochloride and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine) was purchased from BIOMOL Res. Lab. Inc. (PA, U.S.A.). Nicardipine was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). SK&F 94120 (5-(4-acetamido-phenyl)pyrazin-2-[1H]-one), rolipram, cilostazol and zaprinast were gifts from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.), Meiji Seika Kaisha, Ltd. (Tokyo, Japan), Otsuka Pharmaceuticals (Osaka, Japan) and Taisho Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively.

Results Chromatography on a Mono-Q column of the cytosolic supernatant fraction from rat cerebrum yielded three peaks of PDE activity (Figure 1). The enzymatic activity responsible for the second (Peak II) and third (Peak III) peaks was stimulated several fold by the addition of Ca^{2+} /calmodulin (data not shown), suggesting that it was equivalent to PDE I. PDE II and PDE IV, observed in a previous study (Nicholson *et al.*, 1989), were eluted between 0.2 M and 0.6 M NaCl (data not shown).

The first peak (Peak I) of PDE activity, eluted at 0.06 M NaCl, had very high affinity for adenosine 3':5'-cyclic monophosphate (cyclic AMP) [$K_M = 0.109 \pm 0.008 \mu\text{M}$ ($n = 3$)] and relatively high affinity for guanosine 3':5'-cyclic monophosphate (cyclic GMP) [$K_M = 1.78 \pm 0.04 \mu\text{M}$ ($n = 3$)], as shown in Table 1. The ratio of the V_{max} for hydrolysis of cyclic GMP to that for cyclic AMP was greater than one [1.90 ± 0.07 ($n = 3$)]. The hydrolysis of both substrates showed simple Michaelis-Menten kinetics. Ca^{2+} -calmodulin failed to increase the hydrolysis of either cyclic AMP or cyclic GMP. Similarly, cyclic GMP failed to increase the hydrolysis of cyclic AMP.

The effects of various inhibitors of PDEs on the activity in Peak I are shown in Table 1. Nicardipine, a PDE I inhibitor, had a weakly inhibitory effect on the hydrolysis of cyclic AMP [$\text{IC}_{50} > 300 \mu\text{M}$] ($n = 3$). Rolipram and Ro 20-1724, selective inhibitors of PDE IV, had weakly inhibitory

¹ Author for correspondence.

Figure 1 Representative profile after chromatography on a Mono-Q anion-exchange column of cytosolic phosphodiesterase activity from the rat cerebrum. The supernatant from a homogenate of rat cerebrum that had been centrifuged at 100,000 *g* was chromatographed on a Mono-Q column as described in Methods and 1 ml fractions were collected. The concentration of substrate was 1 μ M for both cyclic AMP (●) and cyclic GMP (○). The gradient of 0–0.12 M NaCl is shown by a solid line. These data are representative of data obtained in four separate experiments.

effects on the hydrolysis of cyclic AMP [$IC_{50} > 300 \mu$ M ($n = 3$)]. Similarly, SK&F 94120 and cilostazol, selective PDE III inhibitors, had weakly inhibitory effects on the hydrolysis of cyclic AMP [$IC_{50} > 300 \mu$ M ($n = 3$)]. Zaprinast, a selective PDE V inhibitor, also had a weakly inhibitory effect on the hydrolysis of cyclic GMP [$IC_{50} = 23.5 \pm 3.1 \mu$ M ($n = 3$)]. IBMX had an inhibitory effect on the activity in Peak I, with an IC_{50} value of $26.5 \pm 2.5 \mu$ M ($n = 3$). Cyclic AMP acted as a potent competitive inhibitor of the hydrolysis of cyclic GMP with a K_i value of $0.120 \pm 0.026 \mu$ M ($n = 3$). Cyclic GMP also inhibited the hydrolysis of cyclic AMP in a competitive manner with a K_i value of $2.58 \pm 0.08 \mu$ M ($n = 3$).

Gel filtration indicated that the isoenzyme in Peak I had a molecular mass of 298 ± 8 kDa ($n = 3$).

Discussion This study revealed the presence of a novel isoenzyme of cyclic nucleotide phosphodiesterase in the rat cerebrum. The activity designated Peak I was a cyclic nucleotide PDE with a low K_M that was insensitive to stimulation by Ca^{2+} /calmodulin and cyclic GMP. By contrast to PDE III, this activity had a V_{max} for cyclic GMP that was greater than that for cyclic AMP. With respect to its pharmacological properties, the activity in Peak I was resistant to inhibition by selective inhibitors of PDE III and of PDE IV. These data suggest that the activity designated Peak I represents a novel isoenzyme, which does not easily fit the criteria used in the present classification of PDE isoenzymes (Beavo *et al.*, 1990).

Nicardipine, a potent inhibitor of both intact PDE I and

Table 1 Properties of the novel enzymatic activity from the rat cerebrum

Property	Peak I
Molecular mass (kDa)	298 ± 8
Kinetic data	
K_M cyclic AMP (μ M)	0.109 ± 0.008
K_M cyclic GMP (μ M)	1.78 ± 0.04
V_{max} cyclic GMP	
V_{max} cyclic AMP	1.90 ± 0.07
Sensitivity to inhibitors	
IC_{50} (μ M) ^a	
SK&F 94120	> 300
Cilostazol	> 300
Rolipram	> 300
Ro 20-1724	> 300
Nicardipine	> 300
IBMX	26.5 ± 2.5
Zaprinast ^b	23.5 ± 3.1
K_i (μ M)	
Cyclic AMP (on hydrolysis of cyclic GMP)	0.120 ± 0.026
Cyclic GMP (on hydrolysis of cyclic AMP)	2.58 ± 0.08
Activators	
Ca^{2+} -calmodulin	Not activated
Cyclic GMP	Not activated

Data represent means $\pm$ s.e.mean of results from three separate experiments, each of which was performed in triplicate. For determinations of values of K_M and V_{max} , concentrations of substrate between 0.06 μ M and 100 μ M were used.

^aCyclic AMP at 1 μ M. ^bCyclic GMP at 1 μ M.

trypsin-treated PDE I (Matsushima *et al.*, 1987), had no effect on the activity of the novel isoenzyme. This result suggests that the material in Peak I is not a proteolytic byproduct of PDE I.

The IC_{50} for zaprinast was 30 to 200 fold higher than the values reported for PDE V from the bovine photoreceptor (Beavo *et al.*, 1990) and the canine trachea (Torphy *et al.*, 1989). The hydrolysis of both cyclic AMP and cyclic GMP as substrates showed simple Michaelis-Menten kinetics in each case. Cyclic AMP was a potent competitive inhibitor of the hydrolysis of cyclic GMP by this isoenzyme, having a K_i of the same order of magnitude as its K_M . Cyclic GMP also inhibited the hydrolysis of cyclic AMP in a competitive manner with a K_i of the same order of magnitude as its K_M . These results suggest that the material in Peak I is not a mixture of PDE V and another activity.

In summary, it appears that we have identified an eighth isoenzyme of PDE, which we tentatively designate PDE VIII, from the rat cerebrum.

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